EXHIBIT 31

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Data on protein reserves in ruminant animals are limited. Protein reserves are utilized by high-producing lactating dairy cattle during early lactation. Protein turnover within the body of an animal provides a mechanism for continuous redistribution of amino acids. Nitrogen balance studies have been used by researchers to investigate nitrogen utilization and protein reserves in ruminant animals. This method provides no information on where nitrogen is stored within the body or from which tissues nitrogen is mobilized. Urinary excretion of N^{τ}-methylhistidine appears to be a measure of myofibrillar protein turnover, particularly in rats and humans. It has not been demonstrated that this method can be used as an index of myofibrillar protein turnover in ruminant animals.

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The mechanism and regulation of intracellular protein degradation are still not clearly understood. In this review we discuss various steps which may be presumed to be involved and their likelihood of being rate limiting in the overall process. A number of properties of proteins have been reported to correlate with their degradation rates in vivo, including subunit size, isoelectric point, proteolytic susceptibility, and lipophilic affinity. We propose as a model that the selectivity in turnover rates of proteins is to a large extent based on their rates of translocation to the lysosomal compartment of cells and that this process in turn depends upon their capacity to bind to extracellular or intracellular membranes.

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The degree of coordination between protein synthesis and degradation during protein accretion or diminution in three different types of development transition is examined. Protein turnover patterns in postnatally developing liver, terminally differentiating mammary gland, and in maternal adipose tissue in the perinatal period are presented. Studies on turnover of specific enzymes and resolved protein populations from morphologically (biosynthetically) defined organelle subcompartments are presented. Data are given on the turnover characteristics of monoamine oxidase, pyruvate dehydrogenase, and cytochrome oxidase, as well as proteins in defined mitochondrial subcompartments from rat liver. The turnover characteristics of fatty acid synthetase, casein, and cytosol proteins in rabbit mammary explants in organ culture are presented. Changes in turnover characteristics of lipogenic enzymes, mitochondrial proteins, and cytosol proteins in maternal rat subcutaneous and parametrial adipose tissue around parturition are described. The data from the different model systems are collated and used to formulate a phenomenological hypothesis for protein degradation and its integration with protein synthesis in steady-state and nonsteady-state conditions.

Milk Protein Gene Expression in the Rat Mammary Gland 165

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For the studies of the expression of milk proteins during the functional development of the rat mammary gland and in mammary tumor MTW9, milk proteins were purified, their mRNAs isolated, and the cDNA sequences complementary to these mRNAs cloned in *E. coli* cells. Results of such studies show: (1) rat α-LA is unique in that it is larger than any known α-LAs and is glycosylated; (2) rat milk contains unique whey phosphoproteins not found in other milks; (3) more than one plasmid DNA with differences in the restriction maps have been identified for several of these milk proteins, suggesting either a presence of a family of genes or allelic differences for these proteins; (4) the expression of individual milk proteins is dependent on the functional stage of the gland; (5) there is an inverse relationship between the expression of milk proteins and the methylation of their gene sequences; (6) mammotrophic hormones required for synthesis and stability of milk proteins and their mRNAs, when withdrawn arrest the synthesis of α-LA in the mammary tumor MTW9 at 6 hr or earlier of withdrawal but without any measurable effect on other proteins of the tumor.

Molecular Aspects of Legume Seed Storage Protein Synthesis 187

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The seed proteins of crop plants will provide an ever-increasing proportion of protein in the human diet in the future. Research is currently under way with a variety of crop plants to try to identify the molecular mechanisms that regulate the production of seed proteins. This review, however, will be limited to studies of soybean *(Glycine max)* and common bean *(Phaseolus vulgaris).* The accumulation of storage proteins during seed maturation, the characteristics of the different proteins, and the results of studies of the messenger RNAs encoding the seed proteins have revealed a number of similarities as well as differences between common beans and soybeans. Ongoing research in the isolation and characterization of the genes encoding the seed proteins will provide the much needed background to further explore the molecular mechanisms that regulate the synthesis of these proteins.

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During the development of maize seed large amounts of protein are synthesized and deposited in the endosperm. The largest proportion of this protein consists of the storage protein fraction called zein, which may account for as much as 60% of the total protein. A large number of environmental and genetic factors are known to affect the synthesis of zein proteins. For example, it is well documented that the application of nitrogen fertilizer greatly enhances synthesis of the zein fraction. On the other hand, endosperm mutations such as *opaque*-2 and *floury*-2 reduce the synthesis of these proteins by 50% or more. The biochemical mechanisms by which these factors alter storage protein synthesis have not been identified primarily because little is known regarding the molecular basis for zein synthesis. Our studies have revealed the role of two translational mechanisms involved in enhanced storage protein synthesis in maize endosperm. One of these involves preferential initiation of zein mRNAs, and the other involves cotranslational processing of the zein polypeptides as they enter the lumen of the endoplasmic reticulum.

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Recombinant DNA technology has recently been shown to offer a route to the microbial synthesis of significant amounts of animal, viral, and human proteins. In order to take advantage of this technology, a desired gene must first be obtained and cloned in a suitable host organism. The foreign gene must then be efficiently expressed. In many cases, the foreign gene product must then be isolated. Results obtained through cloning and expressing the chicken ovalbumin gene in microorganisms can be used to demonstrate both the power of this technology and its present limitations.

MOLECULAR MECHANISMS REGULATING THE SYNTHESIS OF STORAGE PROTEINS IN MAIZE ENDOSPERM

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I. INTRODUCTION

A. Seed Storage Proteins

Most plant tissues do not accumulate large amounts of protein; but seeds, especially those of certain cultivated plants, can contain 10 to 50% protein. The majority of this protein is storage protein, so named because it serves as a reservoir of amino acids, nitrogen, and carbon skeletons for the germinating seedling. Although storage proteins of different kinds of seeds have unique characteristics, they share a number of common properties. For example, most of them contain significant amounts, i.e., 30 to 40% amide amino acids, permitting storage of large proportions of reduced nitrogen. Storage proteins usually occur as insoluble deposits in membrane vesicles inside specialized storage cells of the seed. Such deposits have been called "aleurone grains",[1] but since they occur in other seed parts besides the aleurone, it is probably more correct to call them "protein bodies". Storage proteins in protein bodies are quite stable; the length of their half-life is mainly dependent on when the seed is planted.

The storage proteins of cereal seeds are of special interest because of their nutritional importance. They have been the object of intense study for many years, with work on wheat proteins dating from as early as 1745.[2] Most cereal seeds contain approximately 10% protein, and the majority of this is a type of alcohol-soluble protein called prolamine. Prolamine proteins from different cereals have similar solubility properties, but each has its own distinctive amino acid composition and molecular heterogeneity.[3,4] This variation in protein composition results in different functional properties of doughs made from flours of various cereals. As an example, it is possible to make leavened products from some wheat flours, but not from corn meal.

Although there is significant variation in the amino acid composition of different cereal storage proteins, most of them are deficient in one or more of the amino acids essential for human nutrition. Lysine is generally found to be limiting in all of them.[5] A great deal of research has been devoted to the analysis of genetic, physiological, and environmental factors affecting the quality and quantity of cereal storage proteins.[6] One cereal that has been studied extensively in this regard is maize (*Zea mays* L.).

B. Early Studies on Maize Storage Proteins

The maize seed, or caryopsis as it is more properly called, is composed mainly of two structures, the embryo and the endosperm. During seed development the endosperm acts as a storage tissue, accumulating large quantities of starch and protein which are utilized by the embryo during germination.

Endosperm proteins are commonly fractionated based on their solubility in water, 5% saline, 70% ethanol (plus or minus disulfide reducing agents), and alkali, into albumins, globulins, prolamines, and glutelins, respectively.[3,4] The albumins and globulins combined account for about 6% of the total proteins, while the prolamine and glutelins account for around 50% and 40%, respectively, of the total protein.[7] The albumin,

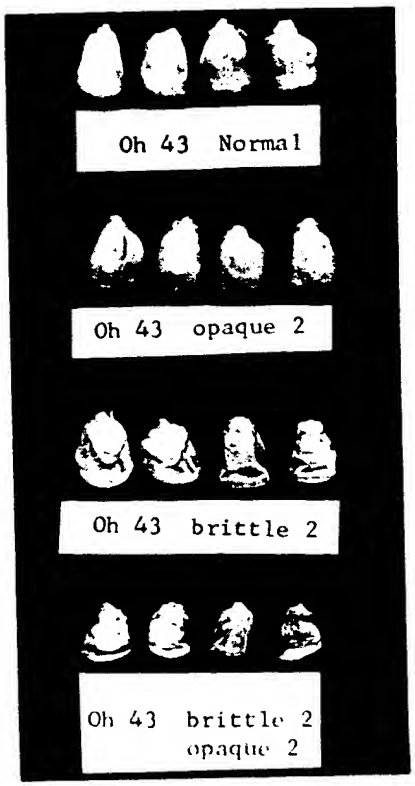

FIGURE 1. Seeds of normal and mutant genotypes of the maize inbred line Oh 43.

globulin, and glutelin fractions are composed of heterogeneous mixtures of polypeptides and are probably derived from a variety of cytoplasmic and membrane proteins. The prolamines, on the other hand, are the functional storage proteins and come from protein bodies inside the rough endoplasmic reticulum (see Figure 5).[8,9]

The amino acid composition of maize prolamines, also called zein proteins, is given in Table 2. Zeins contain large amounts of glutamine, proline, leucine, and alanine. In fact these four amino acids account for nearly 70% of the total. Zeins contain very little of the essential amino acids lysine and tryptophan, and because the amino acid composition of the total seed protein is strongly influenced by the amount of zein, there is an overall limitation of lysine and tryptophan. This can lead to protein deficiency diseases in diets where maize is the principle source of protein.[5]

Several endosperm mutations have been identified that reduce the synthesis of zein proteins and thereby lead to a better nutritional balance of amino acids.[10,11] Children fed corn mean made from the mutant seeds did not develop amino acid deficiencies.[5] But inspite of the improved nutritional quality, the mutants have not been widely utilized. One reason for this is that the reduction of zein proteins changes the baking properties of the meal, resulting in foods that are socially less acceptable. Also significant is the fact that these mutations decrease seed weight, therefore yeild, making them less economical to grow.

Figure 1 and Table 1 illustrate the effect of one of these mutants called *opaque*-2,[10] on the inbred Oh 43.[12] Introduction of the *opaque*-2 mutation leads to a 30% reduction in seed weight, with most of this reflected in the endosperm size. *Opaque*-2 has a significant

Table 1
ZEIN CONTENT OF MAIZE INBRED OH 43 AND MUTANT GENOTYPES

Genotype	Dry wt (mg)		Protein (mg/endosperm)	
	Seed	Endosperm	Zein	Nonzein protein
Normal	267	229	12.6	9.7
Opaque-2	182	148	6.0	10.2
Brittle-2	110	96	4.23	11.8
Brittle-2 *opaque*-2	66	53	0.67	7.3

effect on the protein content, causing about a 50% reduction in the amount of zein proteins. It does not significantly alter the level of nonzein proteins, however. The *brittle*-2 mutation, which affects starch synthesis, also causes a significant reduction in the zein content (Table 1). The combination of *opaque*-2 and *brittle*-2 reduces the zein to about 5% of the normal level. This reduction results in a concomitant increase in the percent of lysine in the total protein. Lysine represents about 1.5% of the amino acids in the normal endosperm, 3.5% in *opaque*-2, and 5.3% in the *opaque*-2 *brittle*-2 double mutant.[13] But it must be stressed that most of this increase occurs as a result of the reduction in zein proteins rather than the synthesis of high lysine-containing proteins. Although these so called "high lysine" mutants do improve the amino acid balance of the seed protein, the problems associated with them have prevented their widespread utilization. They do, nevertheless, provide one means of improving the protein quality of cereal seeds.

In recent years more research has been devoted to the biochemical and genetic characterization of specific storage proteins and to the reactions regulating their synthesis. One of the ultimate goals of this research is to alter specific genes coding for storage proteins so that they synthesize proteins with an increased lysine and tryptophan content.

II. CHARACTERIZATION AND BIOSYNTHESIS OF ZEIN PROTEINS

A. Chemical Characterization

When zein proteins are analyzed by molecular weight using SDS polyacrylamide gel electrophoresis two major and two minor components are resolved (Figure 2). The major ones have molecular weights of 22,000 and 19,000, and the minor ones have molecular weights of 15,000 and 10,000. The *opaque*-2 mutant, in addition to reducing the zein content by 50%, supresses synthesis of the higher-molecular-weight component (Figure 3).

Amino acid analysis of the two major zein components shows they are quite similar (Table 2). There is only slight variation in the composition of most amino acids, although the 22,000 mol wt component has a greater percentage of valine and methionine than 19,000 mol wt component.

When zein proteins are analyzed by isoelectric focusing they show charge heterogeneity within each of the different molecular weight components (Figure 4). In the inbred W64A we estimated that there were four different charged forms of the 22,000 mol wt component and five charged forms of the 19,000 mol wt component; we only detected single forms of the 15,000 and 10,000 mol wt components.[14] Different inbred lines show variation in the pattern of charge heterogeneity,[15] and there is variation in the amino

FIGURE 2. SDS-polyacrylamide gel electrophoresis of zein polypeptides. Zein proteins were dissolved in 0.01% phosphoric acid and separated by Sephacryl S-200 chromatography. Samples in Lanes A and B were taken from the leading and trailing fractions, respectively, of the main protein peak. (From Larkins, B. A., Pedersen, K., Hurkman, W. J., Handa, A. K., Mason, A. C., Tsai, C. Y., and Hermodson, M. A., in *Genome Organisation and Expression in Plants*, Leaver, C. J., Ed., Plenum Press, New York, 1980, 203. With permission.)

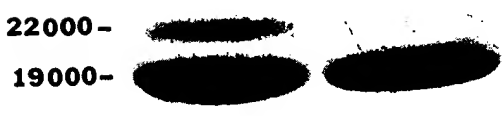

FIGURE 3. SDS-polyacrylamide gel electrophoresis of zein proteins from protein bodies of the maize inbred line W64A and W64A *opaque*-2. Isolated protein bodies were dissolved in buffer (25 m*M* Tris-HCl, pH 8.3, 1% SDS, and 1% 2-mercaptoethanol) and analyzed on a 12.5% polyacrylamide gel.

Table 2
AMINO ACID COMPOSITION OF THE MAJOR ZEIN COMPONENTS

Amino acid	22,000[a]	19,000[a]
Gln	44	38
Leu	39	35
Ala	28	24
Pro	19	17
Ser	14	12
Asm	11	10
Val	11	6
Phe	8	10
Thr	7	6
Ile	6	6
Tyr	6	6
Gly	6	4
Met	3	1
Arg	2	2
His	2	1
Lys	2	1
Lys	Trace	Trace
Total	206	178

[a] Numbers indicate residues per polypeptide.

terminal sequences of the polypeptides.[9,16] So there appears to be both a structural and genetic basis for the variation in isoelectric focusing patterns.

The results of these analyses demonstrate that the zein fraction is composed of a family of homologous polypeptides. Therefore, the synthesis of zein proteins is directed by a developmentally coordinated multigene family.

B. Synthesis and Deposition of Zein Proteins in the Endosperm

Zein proteins are synthesized by membrane-bound polyribosomes during endosperm development,[9,17] and they form insoluble deposits within rough endoplasmic reticulum (RER) membranes (Figure 5). It has been found that many proteins synthesized in this fashion contain a sequence of amino acids at their amino terminus which directs their transport into the RER. This sequence, called a "signal peptide",[18] is simultaneously cleaved by a peptidase during protein transport.

When zein mRNAs are translated in an in vitro protein synthesis system, proteins larger than the native polypeptides are synthesized (see Figure 6).[9,19,20] However, when intact RER vesicles are used to direct protein synthesis in vitro, proteins with molecular weights similar to the native polypeptides are synthesized.[9] These results indicate that some form of protein processing occurs during zein synthesis.

We used *Xenopus laevis* oocytes as a means of studying this processing reaction. The oocytes provide a single-celled in vivo translation system that will not only translate heterologous mRNAs, but also perform post-translational modifications of polypeptides.

A comparison of zein proteins synthesized in the oocyte with those from a cell-free system shows that the oocyte products have the same molecular weight as the native polypeptides (Figure 6). This difference in molecular weight is in accord with the processing of the proteins; furthermore, proteins synthesized in the oocyte have the same amino terminal sequence as the native polypeptides.[20]

Because the enzymes responsible for this processing reaction are on the inner surface of

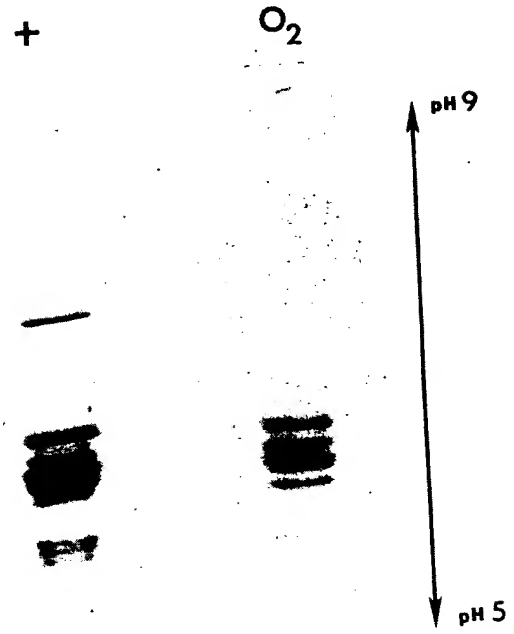

FIGURE 4. Isoelectric focusing analysis of zein proteins from the normal and *opaque*-2 inbreds of W64A.

the RER,[18] we expected that the zein proteins would be compartmentalized inside ER membranes. The results shown in Figure 6 demonstrate that this is the case. Zein proteins are recovered from oocyte membranes extracted with 70% ethanol (Figure 6, Lane 2). Furthermore, the proteins are not susceptible to exogenous protease (Figure 6, Lane 3), indicating that they are inside the vesicles. It is only when detergent is added to break up the membranes, that the proteins are hydrolyzed by the protease (Figure 6, Lane 4).

These results demonstrate that the storage protein mRNAs, and hence the polypeptides, contain the information for directing the compartmentalization of zein proteins within the cell. They also show that this process is not highly specific to maize endosperm cells.

We extended these experiments to determine whether zein proteins associate with one another to form protein bodies inside oocyte membranes as they do in the endosperm.[21] The total oocyte homogenate was fractionated using a linear metrizamide gradient to separate the different subcellular components (Figure 7a). By assaying for marker enzymes and analyzing with the electron microscope we were able to determine the sequential separation of lipid, endomembranes, mitochondria, and yolk platelets. When maize endosperm homogenate was added to the oocyte extract, an additional zone of light-scattering material was detected (Figure 7b), which consisted of zein protein bodies. Therefore, this fractionation procedure clearly separated zein protein bodies from most other oocyte components.

To determine the distribution of zein proteins among the oocyte membrane components, samples were extracted with alcohol or were first treated with protease and extracted with alcohol, and analyzed by SDS polyacrylamide gel electrophoresis (Figure 8). Very little zein protein was associated with the endomembrane fraction (not shown), and there was only a small amount that co-sedimented with mitochondria and yolk platelets (Figure 8, Lanes A and B). The majority of the zein protein was recovered from

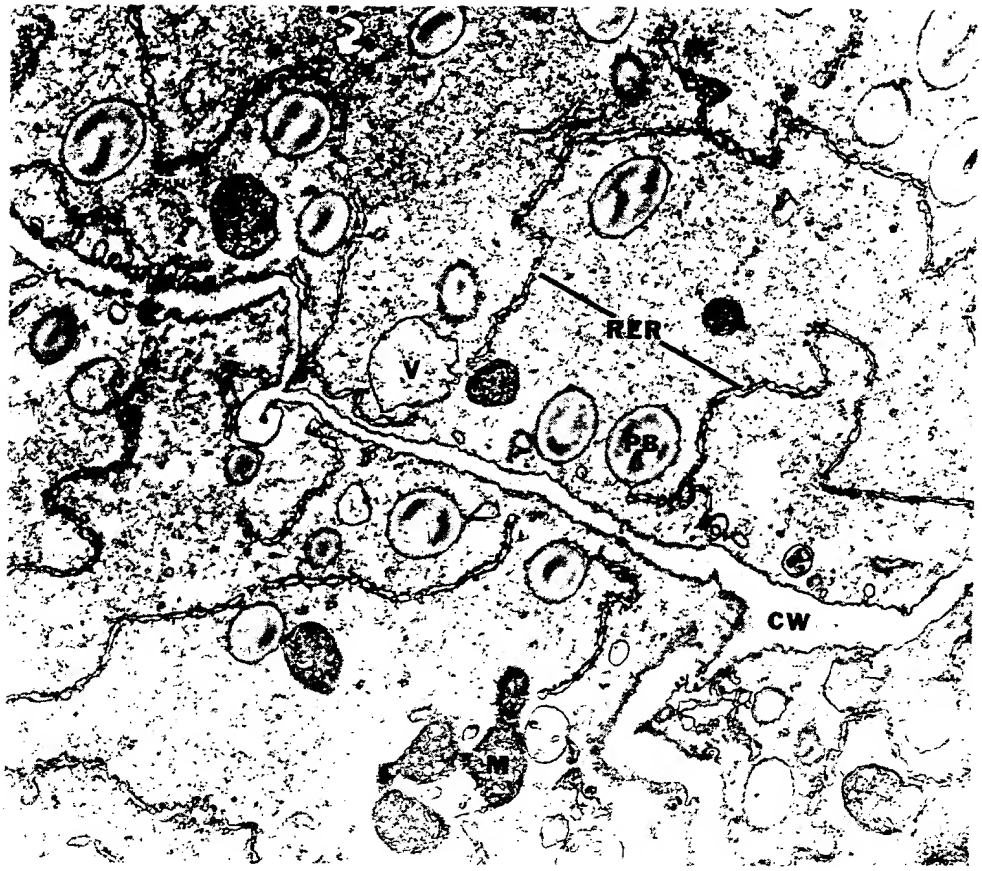

FIGURE 5. Electron micrograph of 19-day maize endosperm cells showing region where storage protein synthesis is occurring. RER, rough endoplasmic reticulum; PB, protein body; M, mitochondrion; CW, cell wall. (From Larkins, B. A. and Hurkman, W. J., *Plant Physiol.*, 62, 256, 1978. With permission.)

the region of the gradient where protein bodies sedimented (Figure 8, Lane C); most of this was inside membrane vesicles, since it was resistant to exogenous protease (Figure 8, Lane C_p).

We analyzed fractions from this region of the gradient by electron microscopy to determine if structures morphologically similar to protein bodies were present. These samples contained several types of spherical inclusions that might contain zein proteins. However, identification of the specific site of zein deposition will require autoradiographic analysis.

We compared the zein composition of "oocyte protein bodies" with that of endosperm protein bodies by two-dimensional (isoelectric focusing/SDS polyacrylamide gel) electrophoresis and found similar sets of polypeptides.[21] We also found that zein proteins synthesized in oocytes had prolonged stability; usually 80% of the radioactivity incorporated into zein proteins during 4 hr of labeling could be recovered 3 days later. Thus it appears that nearly all of the translational and posttranslational reactions of zein proteins occurring in the endosperm can be duplicated in frog oocytes.

C. Preferential Translation of Zein mRNAs

The period of most active storage protein synthesis lasts from around 12 days after pollination until seed maturity at 50 days after pollination.[7] Based upon the amount of

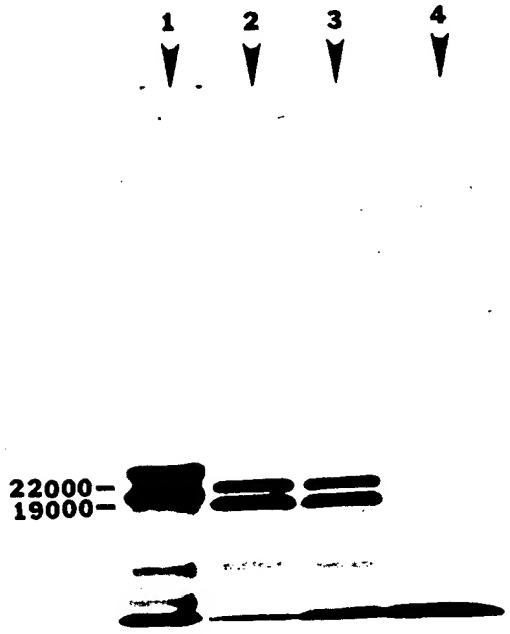

FIGURE 6. Analysis of proteins from membranes of *Xenopus* oocytes after injection of zein mRNAs. Lane 1, zein proteins synthesized in vitro in a wheat-germ cell-free system; Lane 2, 70% ethanol extract of oocyte membranes; Lane 3, oocyte membranes treated with 150 μg/mℓ protease K for 30 min and extracted with 70% ethanol; Lane 4, oocyte membranes treated with 150 μg mℓ protease K and 0.1% SDS for 30 min and extracted with 70% ethanol. (From Larkins, B. A., Pedersen, K., Hurkman, W. J., Handa, A. K., Mason, A. C., Tsai, C. Y., and Hermodson, M. A., in *Genome Organisation and Expression in Plants*, Leaver, C. J., Ed., Plenum Press, New York, 1980, 203. With permission.)

zein accumulated during this time one can calculate that zein synthesis occurs at an average rate of 320 μg per endosperm per day. This is equivalent to nearly 100 ng per cell per hour or 5×10^7 molecules per cell per minute. A number of molecular mechanisms may contribute to this massive synthesis of zein proteins, including enhanced gene transcription, mRNA stability, and mRNA translation. Results of experiments comparing the translation of zein mRNAs with other endosperm and viral mRNAs indicate that preferential initiation of ribosomes on zein mRNAs may play a significant role in this process.[22]

A comparison of the proteins synthesized by membrane-bound and free polysomes of developing endosperm is shown in Lanes A and B, respectively, of Figure 9. The two arrows mark the positions of the two major zein components. When polysomes are used to direct protein synthesis under these conditions, only the preinitiated polypeptides are completed. So this analysis reveals the complexity of proteins normally synthesized in vivo. A comparison of Lanes A and B reveals that the two major zein components are the principle products of the membrane-bound polysomes. There is a small amount of this protein present among the products of the free polysomes;[17] however, they synthesize a much more heterogeneous mixture of higher molecular weight polypeptides. Based on

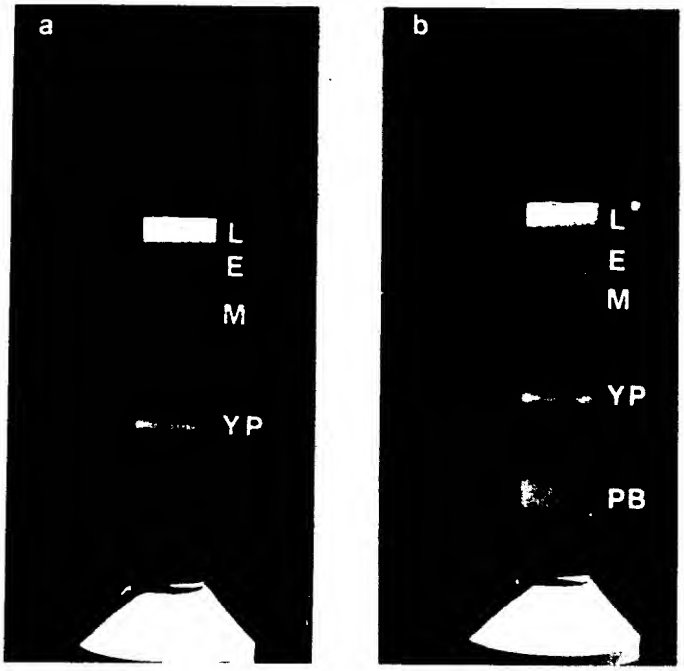

FIGURE 7. Analysis of oocyte and maize endosperm extracts on linear metrizamide gradients. Tissue samples were homogenized in grinding buffer (20 m*M* Tris-HCl, pH 7.6, 0.3 *M* NaCl, 0.06 *M* KCl, 0.01 *M* $MgCl_2$, 0.002 *M* EDTA, and 15% sucrose) and layered onto 10 to 50% metrizamide gradients made in grinding buffer. After centrifugation for 18 hr at 200,000 g, the position of the membrane bands, was visualized with a beam of light. Gradient (a) contained oocyte extract only, gradient (b) contained oocyte and endosperm extracts. L, lipid; E, endomembranes; M, mitochondria; YP, yolk platelets; PB, protein bodies. (From Hurkman, W. J., Smith, L. D., Richter, J., and Larkins, B. A., *J. Cell Biol.*, submitted. With permission.)

this result, we concluded that zein mRNAs are normally segregated between the free and membrane-bound polysomes.[17]

When mRNAs isolated from these two classes of polysomes were translated in vitro, quite different results were obtained. Translation of membrane-bound polysomal mRNAs yielded almost exclusively zein proteins (Figure 9, Lane C); but surprisingly, zein proteins were also major products of the free polysomes (Figure 9, Lane D). Since zein proteins constituted only a small percent of the products when free polysomes were used to direct protein synthesis, this result suggests that zein mRNAs are preferentially initiated in vitro. There are, however, other explanations for this result. For example, nonzein mRNAs could be selectively lost during the isolation of poly(A)-containing mRNAs. Alternatively, the wheat-germ cell-free system may not efficiently initiate or translate mRNAs for the higher molecular weight proteins.

To determine if higher molecular weight mRNAs were lost during the purification of poly(A) RNAs from free polysomes, we translated total free-polysomal RNA in the cell-free system (Figure 9, Lane E). There were some high molecular weight proteins present among these translation productions, but zein polypeptides were still the most prominant. Therefore, mRNAs for the higher molecular weight proteins do not appear to be lost during mRNA purification.

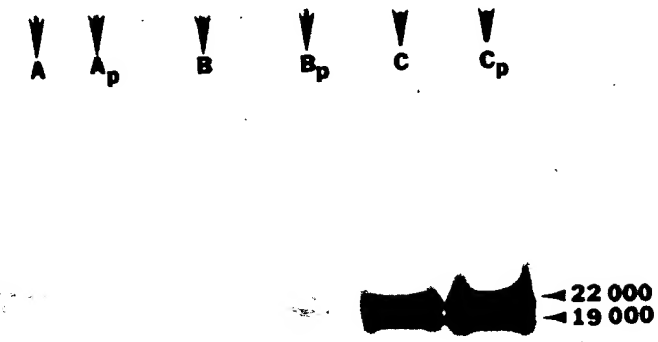

FIGURE 8. Analysis of zein proteins from oocyte membranes fractionated with metrizamide gradients. Samples in Lanes A and A_p were extracted with 70% ethanol from the mitochondrial fraction before and after, respectively protease treatment; samples in Lanes B and B_p were similarly extracted from the yolk platelet faction before and after, respectively, protease treatment; and samples in Lanes C and C_p were isolated from membranes in the region expected for protein bodies before and after, respectively, protease treatment. (From Hurkman, W. J. Smith, L. D., Richter, J., and Larkins, B. A., *J. Cell Biol.*, submitted. With permission.)

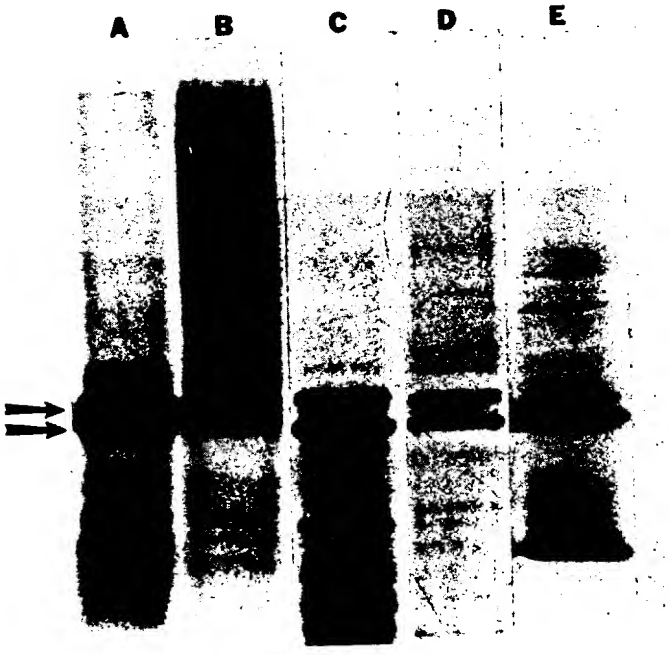

FIGURE 9. Analysis of maize endosperm proteins synthesized in a wheat-germ cell-free system. Lanes A and B show proteins synthesized by membrane-bound and free polysomes, respectively; Lanes C and D show proteins synthesized by mRNAs from membrane-bound and free polysomes, respectively; Lane E shows proteins synthesized by free-polysomal RNA. (From Mason, A. C., M.S. thesis, Purdue University, West Lafayette, Ind., 1980.)

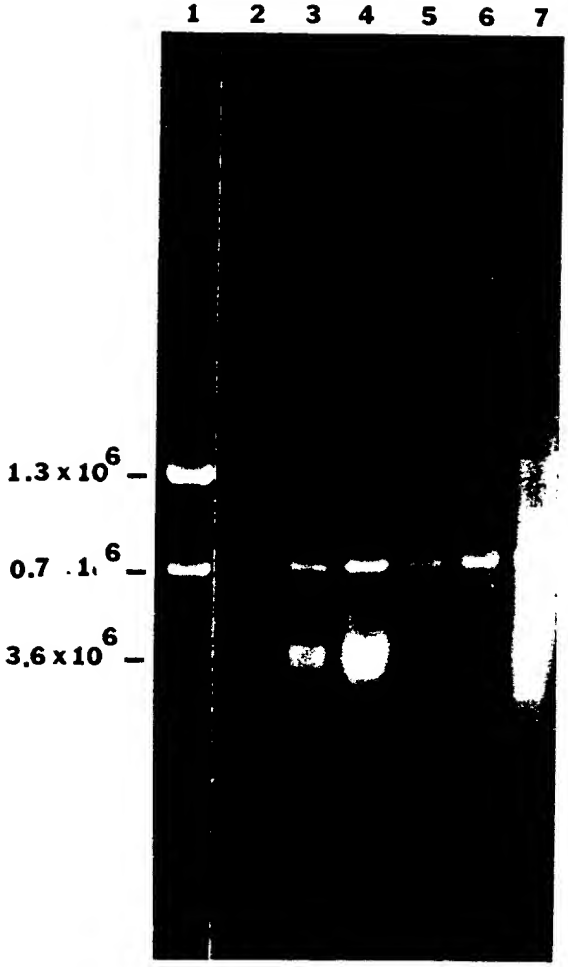

FIGURE 10. Analysis of membrane-bound and free polysomal poly(A) RNAs by methylmercury hydroxide gel electrophoresis. (1) Total ribosomal RNA, 4 μg; (2-4), 3, 5, 10 μg of membrane-bound poly(A) RNA; (5-7), 3, 5, 10 μg free polysomal poly(A) RNA. Molecular weights are indicated to the left.

Additional evidence that these high molecular weight mRNAs were not lost during mRNA purification was obtained by comparing mRNAs from the free and bound polysomes on denaturing methylmercury hydroxide gels. Increasing concentrations of the membrane bound mRNAs (Figure 10, Lanes 2 to 4) revealed a broad RNA band with an average molecular weight of 3.6×10^6 as the most prominent RNA species,[22] although there was a small amount of 18S ribosomal (mol wt 0.7×10^6) RNA contaminating the sample. A similar analysis using increasing concentrations of free-polysomal mRNA (Figure 11, Lanes 5 to 7) indicated that this RNA is much more heterogeneous. There was also some 18S ribosomal RNA in this preparation, but it clearly contained a more complex mixture of higher molecular weight mRNAs.

To determine if these high molecular weight mRNAs could be translated when separated from zein mRNAs, we analyzed a sample on a linear-log sucrose gradient (Figure 11A). After fractionating the gradient, mRNAs were isolated and translated in

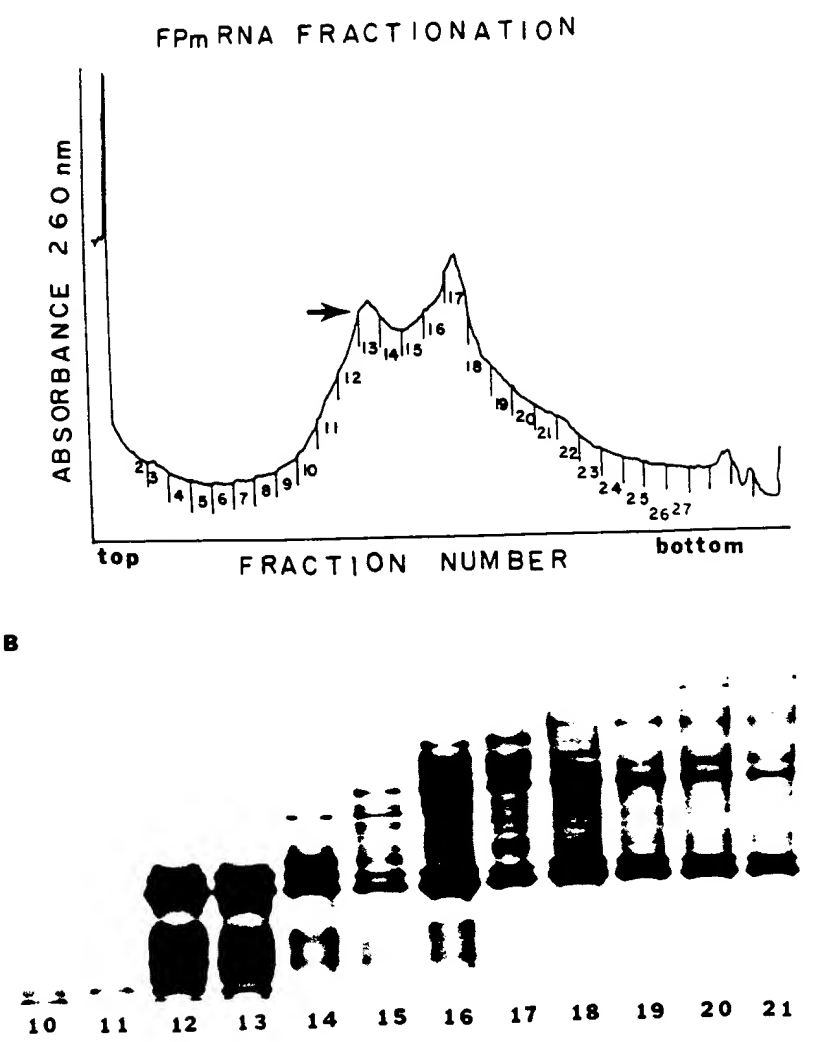

FIGURE 11. Fractionation and translation of free polysomal poly(A) RNA. (A) Approximately 98 μg of RNA isolated from free polysomes by oligo(dT)cellulose chromatography was separated on a 7.5 to 30% linear-log-sucrose gradient, and centrifuged at 25,000 rpm for 14 hr in a Beckman SW41 rotor. The gradient was scanned at 260 nm and fractionated into 0.4-mℓ samples. The position where zein mRNAs sediment in the gradient is indicated by the arrow. (B) Fluorographic analysis of cell-free translation products synthesized by fractionated mRNAs. Numbers refer to the gradient fractions indicated in (A).

vitro. The fluorographic analysis of the translation products shown in Figure 11B shows a concomitant increase in the size of the proteins with increasing mRNA molecular weight. The major zein bands were the predominant proteins synthesized by the small molecular weight mRNAs, and there was evidence of these proteins in all of the gradient samples. Nevertheless, fractions containing high molecular weight mRNAs synthesized large polypeptides, indicating that the mRNAs can be translated in fractions depleted of zein mRNA sequences.

We attempted to determine if zein mRNAs were more efficient at initiating translation

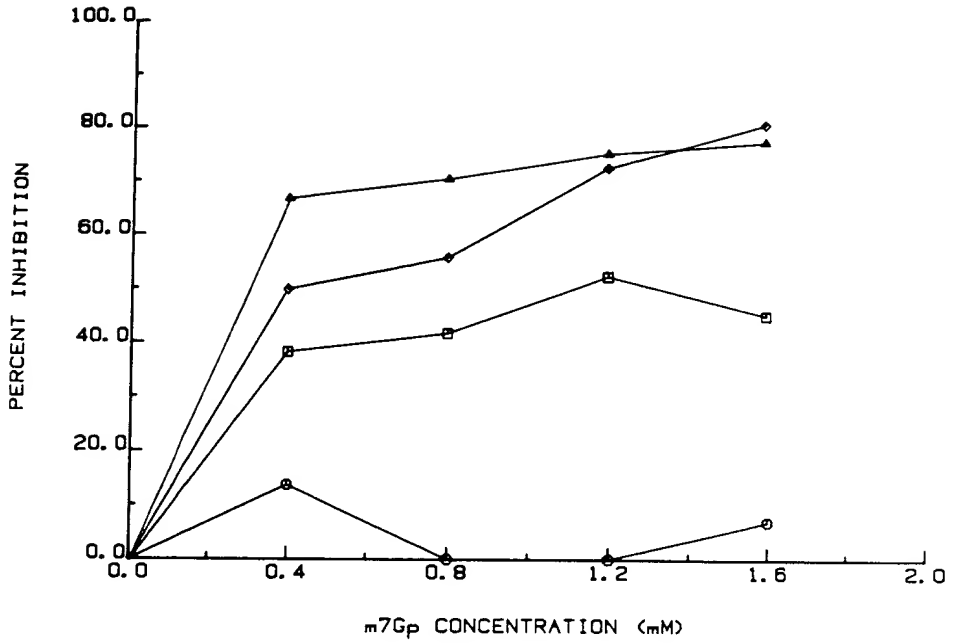

FIGURE 12. Affect of 7-methylguanosine-5′-monophosphate on in vitro translation of zein mRNAs. After establishing optimal RNA concentrations using standard conditions, TMV (▲), STNV (○), free polysomal mRNA (◇), and membrane-bound polysomal mRNA (□) were translated in the presence of increasing concentrations of ^7mG_p.

by comparing the effect of an initiation inhibitor on the translation of the free and bound mRNAs. The 5′ end of most eukaryotic mRNAs contains an inverted terminal nucleotide 7-methylguanosine triphosphate cap, which is thought to play an important role in the binding of the small ribosomal subunit during initiation.[23] It has been found that the cap analog 7-methylguanosine-5′-monophosphate (^7mG_p) inhibits ribosome initiation on most capped mRNAs by blocking specific binding sites on the ribosome.[24] Resistance to translational inhibition by ^7mG_p has also been correlated with selective or preferential mRNA translation.[25]

We determined the effect of ^7mG_p on the translation of free and membrane-bound polysomal mRNAs by measuring the percent inhibition of protein synthesis with increasing concentrations of the analog. As control mRNAs we used tobacco mosaic virus (TMV) RNA, which has a cap, and satellite tobacco necrosis virus (STNV) RNA which is not capped, but is a very competitive mRNA.[25] The results presented in Figure 12 show that the translation of TMV RNA was very sensitive to ^7mG_p; at 0.2 m*M* the translation was inhibited by 80%. STNV, on the other hand, was relatively insensitive to the analog at all concentrations. In these experiments we found STNV RNA to be less sensitive to translational inhibition by ^7mG_p than we previously reported.[26] This may be due to differences in salt concentrations,[21] which have been shown to affect mRNA sensitivity to ^7mG_p.[27]

The translational inhibition of the free and bound polysomal mRNAs was intermediate to the viral RNAs. Significantly, translation of the free polysomal mRNA was more sensitive to the base analog than the membrane-bound mRNA at all concentrations of ^7mG_p. These results might be expected if zein mRNAs, which make up the majority of the membrane-bound mRNAs, were more efficient at forming initiation complexes than free polysomal mRNAs. We examined the types of proteins synthesized

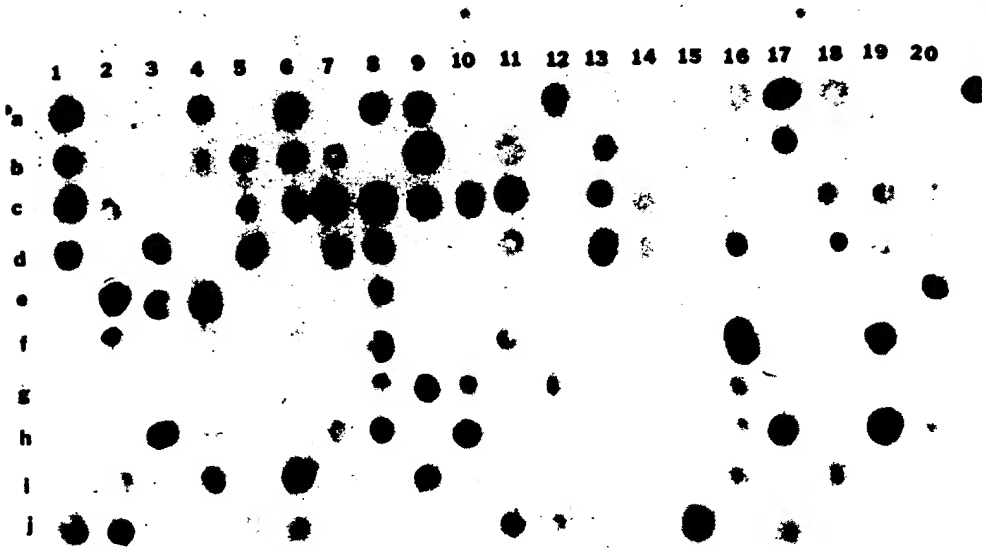

FIGURE 13. Analysis of recombinant cDNA clones of zein mRNAs. Double-stranded cDNAs were cloned into the Pst I site of pBR322 as described by Gordon et al.[28] Bacterial cells containing the recombinant plasmids were lysed on nitrocellulose paper. After baking the DNA, it was hybridized to ^{32}P-labeled cDNA to screen for complementary sequences.

by both classes of mRNA in the presence of increasing concentrations of $^{7}mG_p$, and found that they were not altered qualitatively. Therefore, the inhibition has a general effect on all of the mRNAs.

The results of these experiments are consistent with the conclusion that zein mRNAs are more efficient templates for protein synthesis than other endosperm mRNAs. At the present time it is difficult to assess the contribution of this factor to the overall rate of zein synthesis in vivo. In endosperm cells the rate of protein synthesis would not only be limited by the rate of ribosome initiation, but also by the rate at which signal peptides are bound to RER membranes. It is possible that both of these reactions play a significant role in regulating the rate of zein synthesis.

The fact that zein proteins are synthesized by mRNA from the free polysomes and not by intact polysomes suggests that the mRNAs may exist as ribonucleoprotein particles (mRNPs) or as short polysomes that have not yet attached to RER membranes. If the latter is true, then binding of signal peptides to RER membranes may play a significant role in the regulation of storage protein synthesis.

III. MOLECULAR CLONING OF ZEIN mRNAs AND ISOLATION OF ZEIN GENES

In order to understand the molecular and genetic mechanisms regulating the expression of zein genes, it is necessary to have well-characterized molecular probes for specific zein sequences. Since the zeins are a family of homologous polypeptides, molecular cloning techniques are particularly useful for approaching this problem.

We prepared clones of zein mRNAs by synthesizing complementary DNAs (cDNA)

FIGURE 14. Autoradiograph showing hybridization of zein cDNAs to a nitrocellulose filter-lift of recombinant phage containing maize nuclear DNA. Phage placques showing hybridization were picked with a glass pipet and subplated to obtain pure phage colonies.

with AMV reverse transcriptase. The cDNAs were double stranded with DNA polymerase I and cloned into the Pst 1 site of pBR322 by the dGdC tailing method described by Gordon et al.[29] The recombinant DNAs were used to transform an HB101 strain of *Escherichia coli,* and transformants were screened for growth on tetracycline (tet^+) but not ampicillin (amp^-). Two hundred of the tet^+ amp^- colonies were analyzed by the procedure of Grunstein and Hogness[30] for cDNA inserts using ^{32}P cDNA (Figure 13). Plasmids isolated from colonies showing hybridization were then sized by agarose gel electrophoresis. By this means of selection we identified several potentially full-length cDNA clones; these are presently being characterized for the nature of their cDNA insert.

Genes coding for zein proteins have been isolated from a maize genomic library prepared by Sheldon and Stout in Smithie's laboratory at the University of Wisconsin. The maize library was constructed by ligating Eco R1 partial digests of maize nuclear DNA to the lambda phage charon 4A.[31] We screened the maize lambda library using zein cDNAs (Figure 14) to identify clones containing zein gene inserts. Twelve phage plaques hybridizing to zein cDNAs and cDNA clones were subsequently isolated and are now being analyzed by restriction enzyme mapping for eventual DNA sequencing.

Much remains to be done toward the analysis and characterization of these cloned zein sequences. But eventually they will be powerful tools for analyzing the molecular and genetic factors regulating the synthesis of zein proteins. They may even serve as substrates for new forms of zein genes containing larger amounts of essential amino acids. Improving the amino acid quality of storage proteins using such a recombinant DNA approach could eventually revolutionize plant breeding, providing a method can be developed for reinserting the modified genes back into the genome.

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